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Pluripotent Cell Lines Derived from Common Marmoset (*Callithrix jacchus*) Blastocysts[1]

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ABSTRACT

We report the derivation of eight pluripotent cell lines from common marmoset (*Callithrix jacchus*) blastocysts. These cell lines are positive for a series of markers (alkaline phosphatase, SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81) that characterize undifferentiated human embryonal carcinoma cells and rhesus embryonic stem cells. All eight cell lines had a modal chromosome number of 46; seven cell lines were XX and one was XY. Two cell lines (Cj11 and Cj62) were cultured continuously for over a year and remained undifferentiated and euploid. In the absence of fibroblast feeder layers, these cell lines differentiated to multiple cell types, even in the presence of leukemia inhibiting factor. Differentiated cells secreted bioactive CG into the culture medium and expressed α-CG, β-CG, and α -fetoprotein mRNA, indicating trophoblast and endoderm differentiation. Bioactive CG secretion in differentiating cells was increased substantially in the presence of GnRH agonist D-Trp[6]-Pro[9]-NHEt. When grown at high densities, these cells formed embryoid bodies with a close resemblance to early postimplantation embryos, including the formation of a yolk sac, amnion, and an embryonic disc with an early primitive streak. These results make these pluripotent cells strong candidates for marmoset embryonic stem cells.

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cell lines capable of contributing to derivatives of all three embryonic germ layers even after prolonged culture [1–3]. Mouse ES cells in chimeras sometimes contribute to germ cells, thus providing a vehicle for introducing genetic changes into the germ line [4]. Because homologous recombination allows the alteration of specific loci of the genome, mouse ES cells allow the production of very specific models of human genetic diseases [5]. However, because of the differences between human and mouse development, anatomy, and physiology, transgenic mice can provide only a limited understanding of some human diseases. In addition, the testing of new therapies in transgenic mice is limited by mouse size, life span, and physiology. Transgenic primate models would increase our understanding of the pathogenesis of specific diseases and allow the testing of new therapies. In transgenic primates, therapeutic efficacy for treating degenerative neural diseases, such as Alzheimer's disease, could be assessed not only by morphological and biochemical changes in the brain, but by changes in complex behaviors.

We have recently reported the isolation of ES cells from the rhesus monkey that are immortal, have a stable normal karyotype, and have the potential to differentiate to derivatives of trophectoderm and all three embryonic germ layers [6]. Rhesus monkey ES cells provide a powerful new in vitro model for understanding the differentiation of human tissues, but the reproductive biology of rhesus monkeys makes testing the ability of these cells to contribute to the germ line in chimeras impractical. The rhesus monkey, which is an Old World primate species, has single young, reaches sexual maturity at 4–5 yr, and has an ovarian cycle that cannot be routinely synchronized. The common marmoset, a New World primate species, has more favorable reproductive characteristics for experimental primate embryology, including the natural birth of twins or triplets, an early age at sexual maturity (about 18 mo), and an ovarian cycle that can be synchronized with prostaglandins, thus allowing efficient embryo collection and transfer [7–9].

Here we report the derivation of eight pluripotent cell lines from common marmoset blastocysts that closely resemble rhesus ES cells and human embryonal carcinoma (EC) cells in morphology, growth characteristics, cell surface markers, and in vitro differentiation. Because of the reproductive characteristics of the common marmoset, it will be possible to define the developmental potential of these pluripotent cell lines in chimeras with normal embryos in vivo, initiating exciting advances in experimental primate embryology.

MATERIALS AND METHODS

Embryo Recovery and Cell Line Isolation

For embryo donors, female marmosets greater than 2 yr of age and demonstrating regular ovarian cycles were maintained in groups with a fertile male and up to five progeny. Ovarian cycles were controlled by i.m. injection of 0.75 μg of the prostaglandin $F_{2\alpha}$ analog cloprostenol (Estrumate; Mobay Corp., Shawnee, KS) during the middle to late luteal phase [7]. Blood samples (0.2 ml) were collected in heparinized syringes on Day 0 (immediately before cloprostenol injection), and on Days 3, 7, 9, 11, and 13. Plasma progesterone concentrations were determined by ELISA [10]. The day of ovulation was taken as the day preceding a plasma progesterone concentration of 10 ng/ml or more [8]. Eight days after ovulation, marmosets were lightly anesthetized by the i.m. injection of alphaxalone and alphadolone (Saffan; Glaxovet, Ltd., Uxbridge, UK), and blastocysts were recovered by a nonsurgical uterine flush procedure [9].

Blastocysts were incubated in 0.5% pronase-Dulbecco's

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Modified Eagle Medium (DMEM) while observed under a stereomicroscope. Immediately after zona pellucida dissolution, the blastocysts were washed through two changes of DMEM. To remove the trophectoderm by immunosurgery [11], the blastocysts were exposed to a 1:50 dilution of rabbit anti-marmoset spleen cell antiserum in DMEM for 30 min, washed three times in DMEM, and then incubated in a 1:10 dilution of guinea pig complement (Gibco Labs., Grand Island, NY) for 30 min. After two further washes in DMEM, lysed trophectoderm cells were removed from the intact inner cell mass (ICM) by gentle pipetting, and the ICM was plated on inactivated (3500 rads gamma irradiation) mouse embryonic fibroblasts. Unless otherwise noted, culture medium consisted of 80% Dulbecco's Modified Eagle's Medium (DMEM; no pyruvate, high-glucose formulation, Gibco BRL), with 20% fetal bovine serum (Hyclone Labs., Logan, UT), 0.1 mM β-mercaptoethanol (Sigma Chemical Company, St. Louis, MO), and 1% nonessential amino acid stock (Gibco BRL) [12]. After 7–10 days, ICM-derived masses were removed from endoderm outgrowths, exposed to 0.05% Trypsin-EDTA (Gibco BRL) for 3–5 min, and gently dissociated by gentle pipetting through a micropipette. Dissociated cells were replated on embryonic feeder layers in fresh medium and observed for colony formation. Colonies composed of closely packed cells with high nuclear/cytoplasmic ratios were individually selected, split again, and cultured in the same DMEM-supplemented medium. Early passage cells were frozen and stored in liquid nitrogen [12]. Cell lines were karyotyped with a standard G-banding technique and compared to published karyotypes for the common marmoset [13].

Cell Surface Markers

Cell lines Cj11 and Cj62 cultured on a layer of mouse embryonic fibroblasts were used to examine the expression of cell surface markers. Alkaline phosphatase was detected histochemically after fixation of cells with 100% ethanol, with "Vector Blue" (Vector Labs., Burlingame, CA) used as a substrate, as described by the manufacturer. The SSEA-1, SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81 antigens were detected by immunocytochemistry with specific primary monoclonal antibodies (gifts of Peter Andrews, University of Sheffield, Sheffield, UK) and were localized with a biotinylated secondary antibody and then an avidin/biotinylated horseradish peroxidase complex (Vectastain ABC system, Vector Labs.) [14–16]. NTERA2 cl.D1, a pluripotent human EC cell line (gift of Peter Andrews, University of Sheffield), was used as a negative control for SSEA-1, and as a positive control for SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81 [17, 18]. Mouse ES cells (ES-jt3) were used as a positive control for SSEA-1, and as a negative control for SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81.

Differentiation

Embryo-derived marmoset cells were plated at low density in the absence of fibroblasts on gelatin-treated four-well tissue culture plates (Nunc, Roskilde, Denmark) in the same medium used for initial cell line isolation, but with either 0 or 1000 U/ml of added human leukemia inhibitory factor (LIF; Gibco). The resulting differentiated cells were photographed 8 days after plating.

RNA was prepared by guanidine isothiocyanate-phenol/chloroform extraction [19] from cultures of Cj11 and Cj62 cells grown on embryonic fibroblasts and allowed to differentiate spontaneously for 2 wk after achieving confluence. The mRNAs for α-fetoprotein (αFP), the α- and ß-subunits of CG (CGα and CGβ), and GnRH were detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Primers for the αFP, CGα, and CGβ mRNAs were as previously reported [6]. Primers for GnRH were based on human sequences of the second and fourth exons (5′ primer = (5′) *gggtcgac*TCCAGCCAGCACTGGTCCTATGG; 3′ primer = (5′) *gggtcgac*CTGCCCAGTTTCCTCTTCAATCAG) and amplify a cDNA of 219 bp (lower-case italics indicate nucleotides added to facilitate subcloning) [20]. The identities of the αFP, CGα, CGβ, and GnRH cDNAs were verified by subcloning and sequencing (not shown). Homology of all marmoset cDNAs with the human sequences was > 90%.

To measure the response of CG secretion to a GnRH agonist, Cj62 cells were plated on two four-well plates (Nunc) and allowed to grow to confluence. At confluence, the medium of one plate was supplemented with the GnRH agonist D-Trp^6-Pro^9-NHEt [21] at 0.30 nM; a second control plate was left unsupplemented. Medium was changed every other day for 2 wk, and fresh agonist was prepared from frozen 100-strength stocks at each medium change. Medium from each individual well was assayed for LH/CG activity by a mouse Leydig cell bioassay [22].

For embryoid body formation, Cj62 cells were grown beyond confluence on fibroblast feeder layers and allowed to spontaneously differentiate for 4 wk. One embryoid body was sectioned at 1 μm, rinsed twice with 0.1 M cacodylate buffer, pH 7.0, fixed with Karnovsky's fixative, post-fixed with osmium, and embedded in epoxy. One-micrometer sections were stained with toluidine blue dye and examined under a light microscope; selected ultrathin sections were stained with lead citrate and uranyl acetate, and examined on a Phillips 410 transmission electron microscope.

RESULTS

Eight pluripotent cell lines were derived from marmoset blastocysts, two of which, Cj11 and Cj62, were cultured continuously for over 12 mo; the others were frozen at 3–8 mo of culture for later analysis. These cells had a high nucleus:cytoplasm ratio, prominent nucleoli, and a compact colony morphology (Fig. 1A) similar to, but distinguishable from, that of human EC cells. Cj11 and Cj62 cells expressed a series of cell surface markers (alkaline phosphatase, SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81) that characterize undifferentiated human EC cells and rhesus ES cells (Fig. 2) [6, 16, 18, 23]. Immunostaining for SSEA-3 and TRA-1–81 was weaker than that for SSEA-4 and TRA-1–60, and the intensity varied both between and within colonies. Each of the marmoset cell lines had a modal chromosome number of 46; seven were XX and one was XY (Table 1).

When each of the eight pluripotent marmoset lines was

TABLE 1. Karyotypes of pluripotent marmoset cell lines.

Cell line	Chromosome number	Sex
Cj11.2	46*	XX
Cj25.1	46*	XX
Cj28	46	XY
Cj33	46*	XX
Cj35	46*	XX
Cj36	46*	XX
Cj39	46	XX
Cj62	46*	XX

* Some rare cells with abnormal karyotypes were observed, including fused chromosomes, marker chromosomes, and extra chromosomes.

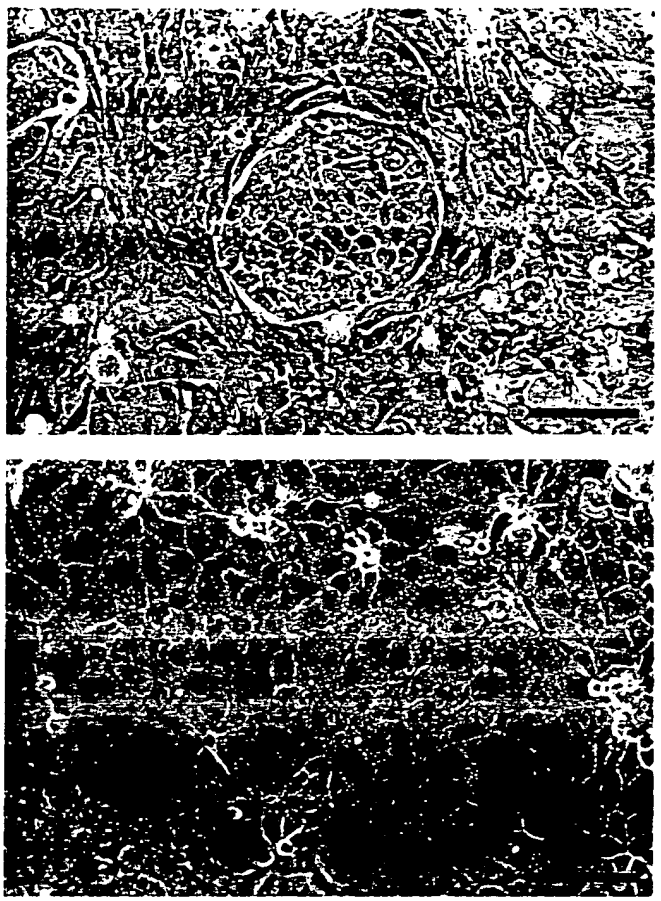

FIG. 1. Colony morphology and in vitro differentiation of cell line Cj62. **A)** Undifferentiated Cj62 cells on a background of embryonic fibroblasts. Note the distinct colony border, high nucleus:cytoplasm ratio, and prominent nucleoli (bar = 100 μM). **B)** Differentiated cells 8 days after Cj62 cells were plated on gelatin-treated tissue culture plastic, with 10^3 U/ml added human LIF (bar = 100 μM).

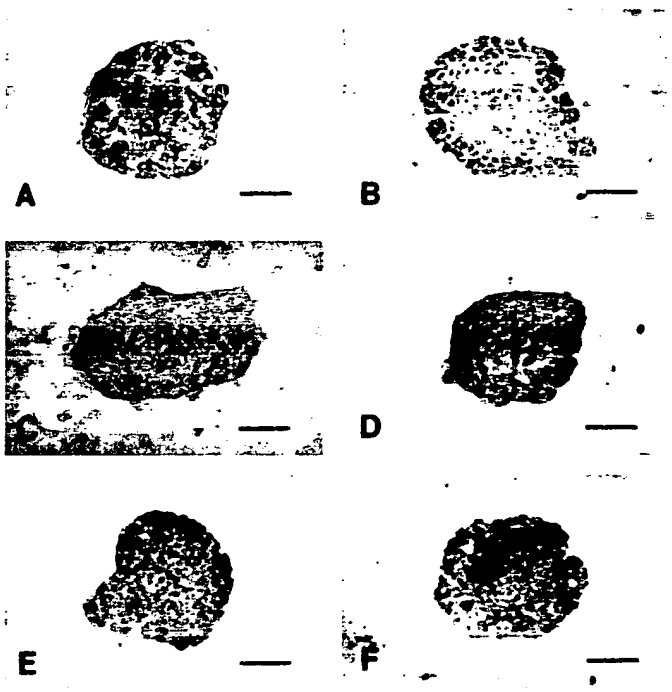

FIG. 2. Expression of cell surface markers by undifferentiated Cj62 cells (bar = 100 μM). **A)** Alkaline phosphatase (Vector Blue substrate). Because no counterstain was used, the fibroblast feeder layer is not visible. **B)** SSEA-1. **C)** SSEA-3. **D)** SSEA-4. **E)** TRA-1-60. **F)** TRA-1-81. For panels **B-F**, detection was with horseradish peroxidase/diaminobenzadine, and positive cells are brown. Counterstaining was with hematoxylin. Although consistently positive, SSEA-3 and TRA-1–81 staining of Cj62 cells was weaker than SSEA-4 and TRA-1–61 staining, and cell-staining intensity varied within and between colonies.

removed from fibroblast feeders, they differentiated into cells of several distinct morphologies, even in the presence of human LIF (Fig. 1B). The cells also differentiated when allowed to grow beyond confluence on fibroblast feeder layers. Among the differentiated cells derived from Cj11 and Cj62, trophectoderm was indicated by the expression of the CGα and CGβ mRNAs detected by RT-PCR (Fig. 3), and by the secretion of bioactive CG into the culture medium (Fig. 4). Differentiated cells also expressed mRNA for GnRH (Fig. 3), and the secretion of bioactive CG increased substantially when differentiating cells were exposed to GnRH agonist (Fig. 4). Endoderm differentiation (probable extra-embryonic endoderm) was indicated by the presence of αFP mRNA, detected by RT-PCR (Fig. 3).

When each of the eight pluripotent marmoset cell lines was grown at high density, over a period of 1–2 wk epithelial cells differentiated and covered the culture dish; the remaining groups of undifferentiated cells contracted into compact balls and then formed embryoid bodies. Over 3–4 wk, some of the embryoid bodies formed a bilaterally symmetric pyriform embryonic disc, an amnion, a yolk sac, and a mesoblast outgrowth attaching the caudal pole of the amnion to the culture dish. Histological and ultrastructural examination of one of these embryoid bodies (formed from a cell line, Cj62, that had been passaged continuously for 6 mo) revealed a close resemblance to an early primitive

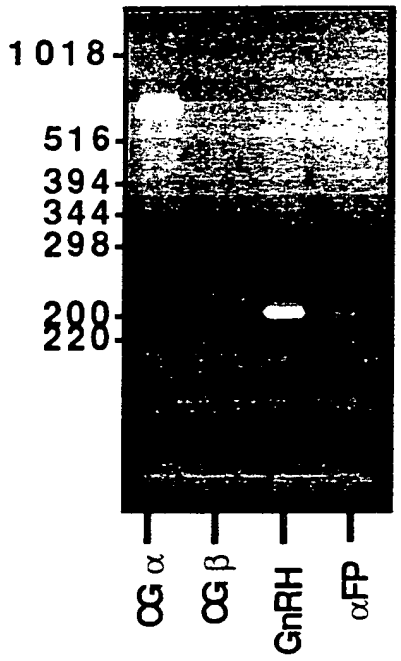

FIG. 3. RT-PCR amplification of mRNAs for CGα, CGβ, GnRH, and αFP from total RNA from pluripotent marmoset cells allowed to differentiate in vitro. Identities of all cDNAs were confirmed by subcloning and sequencing. Negative controls, which contained no template DNA, produced no bands (not shown).

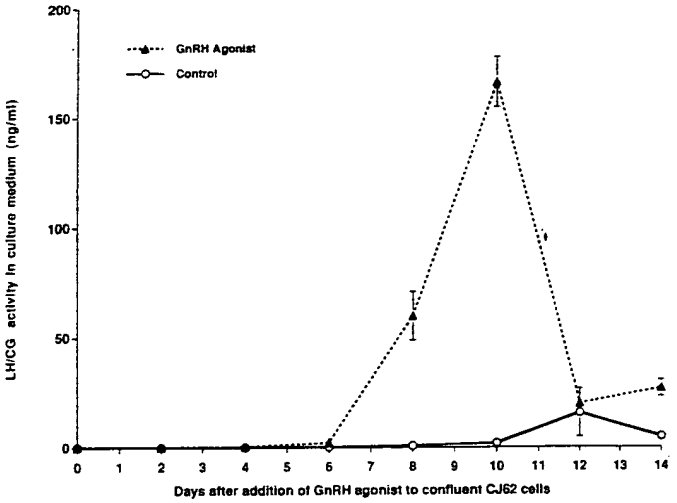

FIG. 4. GnRH agonist D-Trp^6-Pro^9-NHEt responsiveness of CG secretion in differentiating Cj62 cells. LH/CG bioactivity was measured by Leydig cell bioassay in culture medium conditioned by differentiating Cj62 cells. GnRH agonist was added to undifferentiated Cj62 cells at confluence (Day 0); medium was changed every 2 days and supplemented with fresh agonist. Bars represent SEM.

streak-stage embryo (Fig. 5). The embryonic disc was composed of a polarized, columnar epithelial epiblast (primitive ectoderm) layer separated from a hypoblast (primitive endoderm) layer. Electron microscopy of the epiblast revealed apical junctional complexes, apical microvilli, subapical intermediate filaments, and a basement membrane separating the epiblast from underlying endoderm—all features of the normal embryonic disc. In the caudal third of the embryonic disc, there was a midline groove, disruption of the basement membrane, and mixing of epiblast cells with underlying endoderm cells (early primitive streak; Fig. 5). An amnion was composed of an inner squamous (ectoderm) layer continuous with the epiblast, and an outer mesoderm layer.

DISCUSSION

Our criteria for ES cells are as follows: derivation from the preimplantation embryo, immortality, a normal karyotype, and the maintained ability to differentiate to derivatives of all three embryonic germ layers. Contribution to the germ line in chimeras is also a property of some mouse ES cell lines, but originally the term was introduced to distinguish the origin of pluripotent mouse cell lines derived from preimplantation embryos (ES cells) from those derived from teratocarcinomas (EC cells) [3]. Although mouse ES and EC cells are very similar, ES cells generally have a greater developmental potential, a difference thought to be related to the selective pressures of the teratocarcinoma environment that are avoided by the in vitro derivation of ES cells [3].

Several characteristics of the pluripotent marmoset cell lines we have isolated make them strong candidates for ES cells. First, the pluripotent marmoset cells continue to proliferate rapidly for at least 18 mo in continuous culture, and at least some maintain a normal karyotype. Although spontaneously immortal cell lines have been derived from primary cultures of mouse cells, this occurs rarely, if ever,

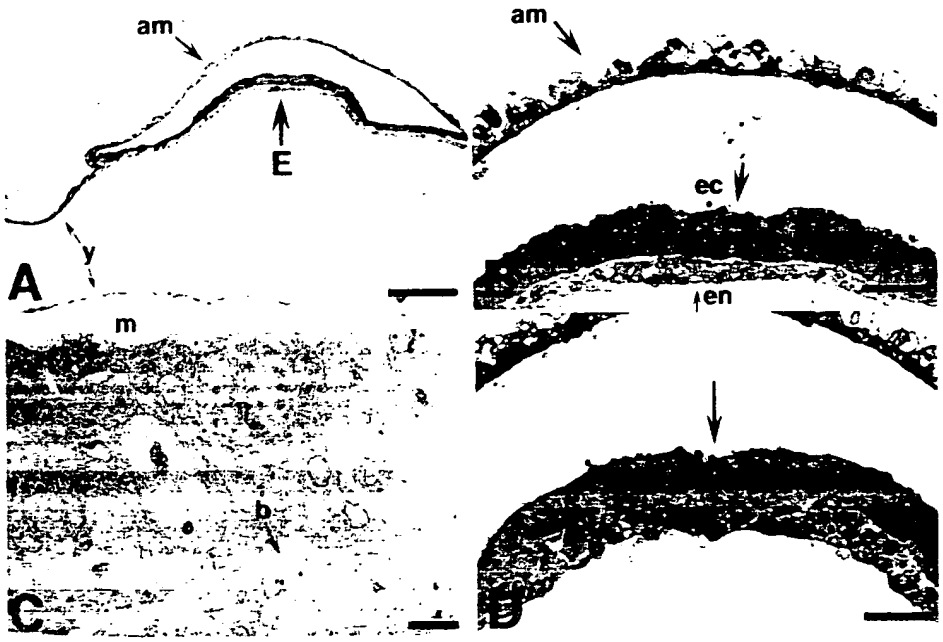

FIG. 5. Embryoid body formed from cell line Cj62 after 6 mo of undifferentiated culture. Cj62 cells were grown to confluence and then were allowed to spontaneously differentiate for 4 wk. **A**) Structures with morphological characteristic of yolk sac (y), amnion (am), and bilayered embryonic disc (E). The yolk sac was spherical, but collapsed during embedding, and the portion of the yolk sac to the right of the photograph was trimmed so the block would fit the diamond knife for sectioning. The embryonic disc was pyriform-shaped with a central groove in its caudal (narrow) aspect, and was connected to the tissue culture plate at its caudal pole by a stalk of mesenchymal cells. (Bar = 200 μM, toluidine blue stain). **B**) Section in cranial 1/3 of embryonic disc. Note that the primitive ectoderm (ec) forms a distinct cell layer from the underlying primitive endoderm (en), with no mixing of cell layers. Note also that the amnion (am) is composed of two distinct layers; the inner layer is continuous with the primitive ectoderm at the margins. (Bar = 50 μM, toluidine blue stain). **C**) Electron micrograph of embryonic disc. Apical microvilli (m) and apical junctional complexes (j) are present in the ectoderm layer, and the basement membrane (b) separates the ectoderm from the underlying endoderm. (Bar = 5 μM, lead citrate and uranyl acetate). **D**) Section in caudal 1/3 of embryonic disc. Note the central groove (arrow) and the mixing of primitive ectoderm and endoderm. This is the approximate level of early primitive streak formation in the normal primate embryo. (Bar = 50 μM, toluidine blue stain).

from primary cultures of somatic cells of primates, which consistently undergo crisis after a characteristic number of cell divisions [24]. Our success in isolating multiple immortal cell lines from both rhesus monkey and marmoset ICMs suggests that, unlike adult somatic cells, the undifferentiated, totipotent cells of the early embryo are immortal; that is, they are capable of unlimited proliferation. A second characteristic of the pluripotent marmoset cells is the expression of SSEA-3, SSEA-4, TRA-1–60, TRA-1–81, and alkaline phosphatase—a combination of cell surface markers previously described only for rhesus monkey ES cells and human EC cells [6, 14–16, 18]. The differentiation of human EC cells results in the loss of SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81 expression, and the earliest lineages to differentiate from the ICM in the human embryo, extra-embryonic endoderm and trophoblast, lack this combination of markers [18]. A third important characteristic of the pluripotent marmoset cells is the potential to differentiate to both endoderm and trophoblast, as the last cells in the mammalian embryo capable of contributing progeny to both these lineages are the totipotent early ICM cells [25]. And finally, the pluripotent marmoset cells differentiate to embryoid bodies with a remarkable resemblance to postimplantation early primitive streak-stage embryos [26].

Pluripotent marmoset cells offer an important new in vitro model for studying the differentiation and function of tissues that differ significantly between mice and primates. For example, the structure and the function of the trophoblast, which forms the outer layer of the placenta, differs dramatically between primates and rodents. Trophoblast secretion of CG in primates, including humans, is central to the maternal recognition of pregnancy. The mouse placenta does not express a CG, and mouse ES cells fail to differentiate to trophoblast or do so infrequently [27]. If the primate corpus luteum is exposed to CG, progesterone secretion is continued and pregnancy is maintained; in the absence of CG, the corpus luteum regresses, progesterone secretion declines, and a new ovarian cycle is initiated. GnRH is expressed in the placenta and has been proposed to have a local regulatory role in CG secretion [28]. The increase in CG secretion we observed in differentiating pluripotent marmoset cells in the presence of GnRH agonist supports a role in CG expression. Further, the dramatic effects observed suggest that GnRH may not only act *on* differentiated trophoblasts but might also be directly involved in the differentiation *of* trophoblasts. GnRH has been shown to be expressed and have biological effects in extrapituitary tissues other than the placenta [29–31], and this may point to a wider role for this regulatory peptide in differentiation and development. Because these pluripotent marmoset cells can be grown indefinitely, prior to differentiation it will be possible to use homologous recombination to modify trophoblast-specific genes, such as CG, GnRH, or their receptors, to help elucidate their function and regulation during and after differentiation.

The pluripotent marmoset cells initiate the formation of all three germ layers in embryoid bodies. If culture conditions can be established that allow efficient, synchronous development of organized embryoid bodies, then it will be possible to use these pluripotent marmoset cells to genetically dissect in vitro the mechanisms controlling early primitive streak formation in primates. We are not aware of primitive streak formation occurring in mouse embryoid bodies, which exhibit a more disorganized development [32]. With our present culture conditions, however, embryoid body formation is asynchronous, and many embryoid bodies develop into simple multilayered vesicular structures without the well-organized structure represented in Figure 5. To date, we have not observed development of embryoid bodies beyond the initiation of primitive streak formation, which is also the approximate stage where intact marmoset embryos degenerate in our culture conditions. To rigorously test the developmental potential of these pluripotent marmoset cells, it will be necessary to provide them with a normal embryonic environment, in chimeras with intact embryos.

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